Multi-response optimization of the extraction and derivatization protocol of selected polar metabolites from apple fruit tissue for GC-MS analysis

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ABSTRACT

Given the complexity of the plant metabolome exhibiting a wide spectrum of physico-chemical properties, finding the best compromise for GC-MS analysis is a challenging exercise. In this study, the GC-MS protocol for extracting and analyzing polar metabolites from apple tissue is optimized. We found pure methanol extraction to be slightly better as compared to the two phase methanol/chloroform/water extraction in terms of introducing less degradation of the extracted metabolites while being comparable in extraction efficiency and repeatability. The methanol extraction was superior to the single phase methanol/chloroform/water extraction in all performance measures. A multi-response optimization based on a desirability function was applied to optimize the derivatization. This procedure allows searching for optimal parameters while simultaneously considering overall detection enhancement of metabolites from various metabolic classes. A short oximation at a high temperature in combination with a low silylation temperature gave results similar to a longer oximation at low temperature in combination with a high silylation temperature. Increasing silylation time from 0.5 h to 2 h resulted in an improvement of the silylation reaction.

Keywords: Apple, Derivatization, Design of experiment, Gas chromatography-mass spectrometry, Metabolomics, Multi-response optimization

1 Introduction

Metabolomics strives for a simultaneous identification and quantification of all metabolites in a biological sample [1]. However, the highly contrasting physico-chemical properties of the metabolome and huge difference in relative abundances, pose a big challenge for developing a single comprehensive protocol capable of meeting this target [2, 3]. While metabolic coverage can be broadened by combining different analytical platforms, such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy, every step of sample preparation and metabolite analysis needs to be optimized for each platform.

A typical GC-MS analysis of polar metabolites consists of quenching, extraction, derivatization, chromatographic separation, metabolite identification and quantification. Intracellular metabolites are usually quenched with liquid nitrogen and extracted using a combination of organic solvents [2, 4]. The ultimate goal of extraction is to maximize the yield and coverage of metabolites in a rapid and reproducible way while minimizing enzymatic, chemical and physical degradation [4]. To achieve these objectives compromises are inevitable. For example, a repeated extraction has positive impact on extraction yield, but will reduce throughput and reproducibility. The choice of extraction solvents depends on whether the compounds of interest are polar, semi-polar or apolar. Several methods are available for extracting hydrophilic metabolites, e.g., 100 % MeOH at 70 °C [5, 6]; 100 % MeOH at 70 °C followed by fractionating into the polar and apolar phase using MeOH:$CHCl_3$:H_2O (2:1:2) [7]; cold MeOH:H_2O extraction [8]; single phase extraction using MeOH:$CHCl_3$:H_2O (2.5:1:1) solution at 4 °C [9] and single phase extraction using MeOH:$CHCl_3$:H_2O (3:1:1) at 60 °C [10]. In addition, chloroform [11], cyclohexane and petroleum ether [12] are commonly used to extract lipophilic compounds.

Solvent temperature, degree of homogenization and solvent-to-solid ratio are important parameters which determine the extraction kinetics and final equilibrium concentration of the extracts. As compared to animal and bacterial cells, plant cells are more difficult to disrupt by mechanical means. Therefore, to increase the extraction rate, mechanical disruptions such as mortar and pestle [13], ball mill [9] and ultrasonication [6] are usually combined with chemical lysis. A higher solvent-to-solid ratio favors the extraction rate by increasing the concentration gradient between solute and solvent [14]. Several studies compared different extraction solvents for plant tissue [10, 15, 16]. Gullberg and co-workers suggested using single phase MeOH:$CHCl_3$:H_2O (3:1:1) extraction for *Arabidopsis thaliana* due to its capacity to extract lipophilic compounds, its simplicity and flexibility for automation as compared to two-phase extraction solvents [10]. However, for the same tissue, t'Kindt and co-workers reported cold MeOH:H_2O (1:4) extraction to provide a large number of metabolites and have better repeatability [16]. On the other hand, Lee and Fiehn found no appreciable difference on composition and extraction efficiency of metabolites between five extraction solvents tested on *Chlamydomonas reinhardtii* [15]. Efficacy and recovery of the metabolites are important extraction performance indicators [17, 18].

A two-step derivatization, methoxymation followed by silylation, is the common method for GC-MS analysis of polar metabolites [4]. Methoxymation is a reaction in which the methoxyamine hydrochloride (MeOX) blocks the carbonyl group of sugars preventing ring formation that would lead to multiple chromatographic peaks. Moreover, it also helps to protect α-keto acids from decarboxylation [19]. The degree of completion of the methoxymation reaction depends on reaction time, and temperature and concentration of MeOX. Different oximation time-temperature combinations have been reported for derivatization of plant tissues, e.g., 30 °C for 90 min [6, 20], 40 °C for 90 min [13] and 50 °C for 30 min [12]. According to Shepherd and co-workers oximation of glucose and fructose is

only partially completed after 90 min at 30 °C. They suggested 4 h at 50 °C as best condition for oximation [21]. Higher temperature and duration ensures the completion of methoxymation, however, this might also result in progressive degradation of heat labile metabolites [10, 21]. In most protocols, the amount of MeOX (20-25 mg ml^{-1}) added to the dried extracts ranges between 50-125 µL. The amount of the initial sample ranges between 100-200 mg, however, the final volume of dried samples largely depends on the extraction protocol and the volume of the aliquot used for drying [12, 20, 22]. Silylation is the principal derivatization technique where the active hydrogen in the functional groups of metabolites is replaced by trimethylsilyl (TMS), increasing the volatility and stability of the metabolites. The most common silylation agents are N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA). The most reported silylation temperatures, durations and amounts of silylation agents for silylation of polar plant extracts range between 30-60 °C, 0.5-1 h and 30-125 µL respectively [10, 12, 20]. BSTFA is the preferred silylation agent because it readily transfers the TMS group, and any derivatization by-products elute with the main solvent peak not interfering with metabolite peaks of interest. Although tert-butyldimethylsilyl derivatives of MTBSTFA have good stability towards hydrolysis, high molecular weight of its hexose derivatives preclude the application of MTBSTFA for GC-MS analysis of sugars [23].

Using a well-structured approach of design of experiments (DOE) [24] experimental parameters affecting the outcome of extraction and derivatization can be optimized in an integrated way. Few studies in metabolomics used DOE to optimize sample preparation for GC-MS analysis, e.g. VOC analysis of *Capsicum chinense* sp. Peppers and apple [25, 26], extraction and MSTFA derivatization of *Arabidopsis thaliana* tissue [10] and extraction and chromatographic separation of adherent cell culture of Clonal β-cells [8]. None of these cases,

considered the gross benefit over all metabolic classes using proper quantitative measures when deciding on the final selection of conditions. Given the highly contrasting physicochemical properties of the metabolome, a multi-response optimization taking into account the different responses of the different individual metabolic classes is the preferred way to find the best compromising set of parameters for GC-MS analysis. This procedure ensures the gross enhancement over all metabolic classes. The objective of this paper was to optimize extraction and derivatization steps of GC-MS analysis of apple polar metabolites using DOE in combination with a multiple-response criterion.

2 Experimental

2.1 Materials

2.1.1 Preparation of standard mix

For the optimization of derivatization, a stock solution of standard mixture of 20 metabolites (200 ng/µL) was prepared consisting of amino acids, organic acids, sugars, sugar phosphates and sugar alcohols representative for apple tissue. Samples were prepared by drying 50 µL of the prepared solution under a stream of nitrogen. The list of all metabolites used for preparation of the standard mixtures and metabolites extracted from apple tissue with m/z used for quantification and their retention index is presented in Table 1. Metabolites are mentioned with their underivatized name throughout the manuscript, not with their derivatives. For simplicity, those metabolites with only one derivatized form are referred to by their underivatized name throughout the manuscript. Only aspartic acid and isoleucine, that showed multiple derivatives, are referred to by their derivatized name.

Aspartic acid, citric acid, fructose, glucose-6-phosphate, pyruvic acid, quinic acid, succinic acid and xylitol were purchased from Acros Organics (Geel, Belgium). Asparagine, dehydroascorbic acid, glutamic acid, stearic acid, isoleucine, glutamine, sucrose, α-

ketoglutaric acid, γ-Aminobutyric acid, cysteine, glucose, phosphoric acid, 2-phosphoglyceric acid and methoxyamine hydrochloride were obtained from Sigma-Aldrich (St. Louis, USA). N,O-bis(trimethylsilyl)-trifluoroacetamide and methyl stearate were purchased from Supelco (Bellefonte, USA)

2.1.2 Preparation of apple sample

The apple sample was prepared by grinding frozen apple (*Malus x domestica* Borkh., cv. "Jonagold") tissue into a fine powder at 20 Hz for 1 min (Retsch Mixer Mill model MM 200).

2.2 Apparatus

Metabolites were analyzed in 6890N GC (Agilent Technologies, Santa Clara, USA) coupled with a 5973 network mass selective detector and equipped with MPS2 multipurpose auto sampler (Gerstel, Mülheim an der Ruhr, Germany). The GC was fitted with an HP-5MS capillary column of 30 m x 0.25 mm x 0.25 µm (Agilent, USA). The samples were volatilized at 220 °C in 4 mm internal diameter deactivated tapered focus liner containing quartz wool (SGE Analytical Science, Australia). Helium was used as a carrier gas with a flow rate of 1 ml/min. The detector was operated in EI mode with the transfer line maintained at 250 °C. The MS source and quadrupole temperatures were at 230 °C and 150 °C respectively.

2.3 GC-MS analysis

For metabolite analysis, 1 µL of derivatized sample was injected into GC-MS. Less abundant metabolites were analyzed using a pulsed-splitless injection while abundant metabolites were analyzed using split injection. In pulsed splitless mode, the inlet pressure was kept at 20 psi for 0.3 min, and reduced to 7.22 psi afterwards. The oven temperature was initially held at 50 °C for 1 min, ramped to 310 °C by 10 °C/min and held for another 13 min. The split ratios of 1:20 and 1:500 were used to analyze the standard mixtures and most abundant metabolites of

151 the apple tissue respectively. For split injection, the oven temperature was held at 120 °C for 1
152 min and ramped at 10 °C/min to 300 °C, and held at this temperature for 6 min.

153 **2.4 Experimental design**

154 Response surface methodology (RSM) based on a Box-Behnken design (BBD) with three
155 levels and six factors (Table 2) was applied [27]. BBD is highly efficient for experiments with
156 high number of factors [28]. At this stage, the levels of the factors were selected to cover a
157 broad range of derivatization parameters. Using Unscrambler X 10.1 (CAMO Software AS,
158 Oslo, Norway), 54 experimental conditions (Supplemental Table S1) located on the
159 hypersphere equidistant from the center points were generated. The total volume of
160 derivatized sample in all conditions was adjusted to 250 µL by adding hexane as needed.
161 Normalized peak areas of 30 metabolites and derivatization artifacts were used as response
162 variables.

163 A second order polynomial (eq. 1) consisting of linear, quadratic and first order interaction
164 terms was fitted to the measured individual response variables.

165 $$y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^{\,2} + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon \qquad (1)$$

166 where y is one of the response variables, x_i represent the derivatization variables,
167 $\beta_o, \beta_i, \beta_{ii}, \beta_{ij}$ are the regression coefficients for intercept, linear, quadratic and interaction
168 terms respectively, k denotes the number of variables and ε represents the remaining
169 unexplained error. The regressions coefficients were estimated by the method of multiple-
170 least square regression that finds the regression coefficients by minimizing the sum of squares
171 of the errors [29].

172 The significance of the overall model, and of each regression coefficient was assessed by
173 analysis of variance (ANOVA). Insignificant model terms were removed by using backward

elimination procedure [30]. The coefficients of the model equations after backward elimination of non-significant terms and the model diagnostics are given in Table 3. Next, the prediction power of the models was assessed based on the significance of the regression models ($p < 0.05$), lack-of-fit ($p > 0.05$), coefficient of determination (R^2) and adjusted R^2 and adequate precision. The lack-of-fit test measures the adequacy of the quadratic models by comparing the variation due to fitting to the variation due to analytical error. An adequate precision value higher than 4 indicates sufficient signal to noise ratio to explore the design space [31]. In addition, plot of normal residuals, residuals vs. predicted, residuals vs. run and Box-Cox analysis were used to diagnosis for abnormalities, trends and normality of the residuals. Based on the Box-Cox analysis, responses deviating from the normal distribution were transformed with power function. Finally, because of its proven versatility, multi-criterion response surface optimization based on the Derringer's desirability function was employed [32, 33]. The function transforms the response of each variable into a desirability score (d) ranging between 0 (completely undesirable) and 1 (entirely desirable). The function takes different forms depending on the optimization criterion in use: maximizing, minimizing or attaining a fixed target. The desirability function for maximizing response variables takes the form of eq. 2.

$$
\begin{aligned}
d_i &= 0 && \mathrm{y}_i \le y_{i,\min} \\
d_i &= \left[\frac{\left(y_i - y_{i,\min}\right)}{\left(y_{i,\max} - y_{i,\min}\right)} \right]^{w_i} && y_{i,\min} < y_i < y_{i,\max} \\
d_i &= 1 && \mathrm{y}_i \ge y_{i,\max}
\end{aligned}
\qquad (2)
$$

Where $y_{i,\min}$ and $y_{i,\max}$ are the minimum and maximum desired levels of each individual response variable which corresponds, in this study, to the highest and the lowest normalized

peak areas of the metabolites in all design runs. Responses below $y_{i,\min}$ were assigned 0 desirability while responses above $y_{i,\max}$ were assigned a desirability of 1. Between $y_{i,\min}$ and $y_{i,\max}$, the desirability increased linearly by assigning a weight (w_i) of one. The desirability function for minimizing took the reflected form of the maximization function. Subsequently, the optimization criterion was set to maximize the normalized peak areas of all metabolites except hexose sugars, and minimize the normalized peak areas of underivatized amino acids and derivatization artifacts. As at higher oximation temperatures, the hydrolysis of sucrose into glucose and fructose was observed, we opted to rely on maximizing the response of sucrose without using the responses of glucose and fructose as optimization criteria. The individual desirabilities were then combined into a global desirability (D) using the geometric mean (eq. 3)

$$D = \left(\prod_{i=1}^{n} d_i^{r_i} \right)^{1/\sum r_i} \qquad (3)$$

where r_i denotes the importance given for a particular response. Equal importance was given for all metabolites by assigning r_i values of 1. The global desirability increases with more metabolites reaching their desired values. Finally, a search algorithm was applied to find a set of parameters which maximizes (D). The search algorithm returned a list of factor combinations and their respective desirability scores. Based on this result and given our objective of optimizing extraction and derivatization on apple tissue, a small range of derivatization parameters were selected and the optimization procedure outlined above was repeated on apple extract. Additionally, minimizing the total processing time was used as optimization criteria by adding it to the list of response variables. Multi-response optimization was done using Design Expert software, version 8.0 (Stat-Ease, Inc., Minneapolis, USA).

2.5 Extraction solvent selection

An ideal extraction method produces the metabolic profile of the extract exactly similar with the original sample. This consists of complete extraction of metabolites and minimum degradation or inter-conversion between the metabolites. Besides, due to the high throughput nature of the metabolomics study, the method should be easy to work and be highly repeatable. Recently, new methods have been suggested to quantitatively determine the extraction efficiency and recovery of the metabolite during extraction [17, 18].

To select the best extraction solvent for apple tissue, three most widely used extraction protocols for plant materials were evaluated, using the tissue powder prepared from 'Jonagold' apple. Solvent ratios are given in volumetric units. Five replicates were used for each method. The recovery of metabolites was calculated by comparing the response of six samples spiked with a mixture of standards before and after extraction [17].

Hot methanol extraction (HS-PM): This method followed the hot methanol extraction used for tomato fruit [5]. 1400 µL of pre-chilled MeOH (-20 °C) was added to 200 mg apple tissue and extracted at 70 °C (15 min, 1400 rpm) in a Thermomixer comfort (Eppendorf AG, Germany). Subsequently, the extract was centrifuged for 20 min at 14,000 rpm in an Eppendorf centrifuge 5417R (Eppendorf AG, Germany). 200 µL of the extract was dried under a stream of nitrogen (Stuart, Staffordshire, UK).

Two-phase chloroform:methanol:water extraction (HT-CMW): The method described by Lisec et al. was used with minor modification [7]. First, 680 µL of MeOH was added to 200 mg of apple tissue and extracted at 70 °C for 15 min in a Thermomixer comfort (Eppendorf AG, Germany). Then, 340 µL of $CHCl_3$ and 680 µL of water was added to the mixture, vortexed for 10 s, and centrifuged for 20 min at 14,000 rpm in an Eppendorf centrifuge 5417R

(Eppendorf AG, Germany). 194 µL of the extract was dried under a stream of nitrogen (Stuart, Staffordshire, UK).

Single-phase chloroform:methanol:water extraction (CS-CMW): This method followed the single phase chloroform:methanol:water extraction as described by Weckwerth et al.[9]. 1400 µL of single phase $CHCl_3$:CH_3OH:H_2O (2:5:2) was added to 200 mg of sample and extracted for 30 min at 4 °C in a Thermomixer comfort (Eppendorf AG, Germany). The extract was centrifuged at 14,000 rpm for 20 min in an Eppendorf centrifuge 5417R (Eppendorf AG, Germany). 200 µL of the extract was dried under a stream of nitrogen (Stuart, Staffordshire, UK).

2.6 Compound identification

Compound identification was performed using Agilent MSD Chemstation software (Agilent Technologies, Santa Clara, USA) by comparing the acquired spectra with Agilent Fiehn Metabolomics Library, NIST98 (National Institute of Standards and Technology, Gaithersburg, MD, USA) and an in-house built library. Deconvolution of the raw chromatogram was done using Automated Mass spectra Deconvolution and Identification System (AMDIS). Next, peak areas of metabolites were quantified by integrating the respective peak areas of quantifying ions. The peak areas of metabolites in reference standard mix were normalized by the peak area of the internal standard methyl octadecanoate. For apple tissue, two internal standards (henceforth IS) were used; phenyl-β-D-glucopyranoside for the sugars and 3-(4-Hydroxyphenyl)propionic acid for the other compounds. These IS's were selected because they are not naturally present in the apple tissue, and they are not co-eluting with other metabolites. The IS's were added after extraction prior to drying and were used for correcting the small variation during derivatization and GC-MS analysis. Two different internal standards were used to represent different derivatization properties of sugars and acids.

Quality control samples were included with each injection sequence of 20 samples. These included a solvent blank (hexane), a method blank (solvents used for extraction and derivatization), a reference standard mixture, and a Grob mixture [34]. The inlet liner and septum were visually inspected for dirt in the liners and puncturing damage of the septum. The liner and the septum were changed every 40-50 injections.

2.7 Repeatability & stability

Variation during pooling of samples, sample preparation steps (weighing, pipetting, quenching, extraction, concentration, derivatization) and the applied analytical methodology (chromatography, detection and quantification procedures) influence the repeatability of the GC-MS method. Repeatability of the final selected method was evaluated from eight replicate samples prepared from a single homogeneous tissue sample. Stability of derivatized samples was assessed by injecting the same sample repeatedly over a period of 48 h.

3 Results & discussion

3.1 Derivatization

We used a two-step approach for optimizing the derivatization of polar metabolites extracted from apple tissue by maximizing the completion of the reaction in the shortest possible time while minimizing the degradation of metabolites. First, the effect of oximation and silylation temperature, time, and volume of MeOX and BSTFA on the derivatization of the standard mixtures was explored over a broad range of these parameters. Using the standard mixture enabled addition of less abundant apple metabolites, and to trace the formation of derivatization artifacts which could be otherwise difficult to identify. Based on the results from this study, narrow bands of the parameters were selected, and the optimization was further refined on apple extract.

All metabolites added to the standard mixture were properly detected and, under certain conditions, pyroglutamic acid (5-oxoproline), phosphoric acid, cystine and five unknown compounds which were not part of the standard mixture were detected as well. Even though 5-oxoproline is a natural product in fruits which can be synthesized by either enzymatic or chemical cyclization of glutamic acid [35], it can also be formed as a derivatization by-product of glutamic acid [36]. Phosphoric acid could be formed from degradation of glucose-6-phosphate or 2-phosphoglyceric acid. Higher oximation temperature and duration increased the formation of 5-oxoproline, while it concomitantly decreased glutamic acid content (Fig. 1). Similar conditions also induced the formation of phosphoric acid. Moreover, low oximation temperatures and short durations reduced the oxidation of cysteine into cystine. When optimizing the derivatization conditions it is important to select conditions which minimize the incidence of these artifacts.

The interactions of the derivatization parameters were studied from the contour plots. The results show that, for most compounds, a clear interaction between the various derivatization parameters exists (Fig. 1-3). However, the derivatization result of succinic acid, citric acid, quinic acid and xylitol was less influenced by the derivatization conditions.

Metabolites were differentially affected by oximation temperature and duration showing either positive, negative or negligible effects (Fig. 1). Higher oximation temperature and longer duration increased the relative response of isoleucine 2TMS and GABA. Higher oximation temperature is speculated to enhance derivatization efficiency by increasing solubility of metabolites [10]. In contrast, cysteine, glutamine and dehydroascorbic acid were readily susceptible to higher oximation temperature and duration. Shepherd and co-workers found the breakdown of ascorbic acid into dehydroascorbic acid and 2,3-diketogulonic acid at oximation temperature of 50 °C [21]. On the other side, long term low temperature oximation was favorable for α-ketoglutaric acid, glutamic acid and asparagine, while either long term

low temperature or a short term high temperature oximation were favorable for pyruvic acid and 2-phosphoglyceric acid. α-keto acids such as pyruvic acid and α-ketoglutaric acid are susceptible to higher oximation temperature [10, 23]. The relative responses of fructose and glucose were positively associated with oximation temperature and duration but similar conditions also decreased the relative response of sucrose. High oximation temperature and duration is known to result in the hydrolysis of sucrose into fructose and glucose [10] indicating that the increase in hexose sugars most likely resulted from sucrose breakdown instead of from a direct effect of the derivatization.

Most amino acids formed a dominant peak of monosilylated amine, but partially derivatized amino acids were also detected at low volumes of BSTFA and MeOX (Fig. 2). For example, the relative response of partially derivatized aspartic acid (aspartic acid 2TMS) was negatively correlated with the volume BSTFA and MeOX added. High volume of BSTFA enhanced the derivatization of isoleucine 2TMS and glutamic acid as well. Gullberg and co-workers similarly found high association between the volume of MSTFA and the number of detected peaks [10]. Pyridine, in which MeOX is dissolved, is known to catalyze the silylation reaction [19]. The disilylated amines of the amino acids were not detected, even after 6 h of silylation. This suggested that the kinetics of formation of disilylated amines of these amino acids is a slow process. Conversely, BSTFA and MeOX volumes significantly enhanced derivatization of monosilylated amines. Interestingly, BSTFA volume had negligible effects on most organic and α -keto acids including pyruvic acid, succinic acid, α-ketoglutaric acid and dehydroascorbic acid. This is in line with the finding of Koek who showed that organic acids and sugars need relatively low volumes of silylating agent as compared to amino acids [37]. The reactivity of TMS groups towards nitrogen in amino acids is lower than that to oxygen in organic acids, having lower number of unshared electrons, higher steric hindrance and

transition state energy [37]. MeOX volume was positively correlated to most amino acids, but negatively associated to 2-phosphoglyceric acid, glucose-6-phosphate and quinic acid.

Hexane was added to maintain fixed the volume of the derivatizing agents used for all runs. Hexane is an apolar solvent which can solubilize the derivatized metabolites, and does not have high influence on the derivatization performance of the metabolites. In line with this, previous researches have shown that the effect of non-polar solvents on derivatization performance is minimum as compared to the effect of derivatizing agents. For example, Gullberg and co-workers found no significant effect of heptane on derivatization performance of diverse metabolic classes [10]. Similarly, Danielsson and co-workers found no appreciable effect of silylation solvent composition on derivatization performance of MBSTFA derivatives [23]. Besides this, hexane does not affect the chromatographic performance, as hexane elutes with the other solvents much before the metabolites.

Low silylation temperature (< 50 °C) and longer silylation time (> 4 h) was beneficial for most compounds (Fig. 3). However, silylation temperature had negligible effect on α-ketoglutaric acid, dehydroascorbic acid, cysteine, citric acid and pyruvic acid. On the other hand, long term high temperature silylation was beneficial to stearic acid and glucose-6-phosphate.

3.2 Multi-response optimization on a standard mixture

The effect of derivatization parameters on metabolites is complex and varies across different chemical groups (Fig. 1-3). What is considered an ideal condition for one chemical category might be unsuitable for another group. It is therefore essential to compare the performance of derivatization protocols based on an overall score which combines the response of all metabolites into a single quantity.

To this end, the global desirability was used to search for optimal derivatization conditions by using geometric mean of the individual desirability's index of the metabolites (*d*). Using the geometric mean ensures that the selected condition fulfills at least the minimum desired value of all responses, and most importantly, *D* increases as most metabolites come close to their desired value. For example, if the selected derivatization condition would favor few metabolites with high d but many metabolites with low *d*, the overall *D* would still be very low. Similarly, the conditions with metabolites having equal but low *d*, yields a low *D* as well. In these cases, neither scenarios are desirable. In optimizing the derivatization for metabolomics analysis, it will be preferable to maximize the degree of conversion of as many metabolites as possible, towards high *D*, so that the selected conditions ensures the overall optimal derivatization performance.

Low oximation temperature (< 50 °C), medium silylation temperature (50-60 °C) for long durations (> 4 h), and higher BSTFA and MeOX volumes (above 87 µL) were predicted as the most desirable derivatization ranges (Fig. 4).

Long silylation durations (> 4 h) were needed to ensure adequate derivatization of slowly reacting metabolites. If these metabolites are not sufficiently derivatized, the reaction might proceed while the samples are waiting their turn for injection. Depending on the number of injections in a batch a run order based bias can be induced as there can be a 1-20 h time difference between analyses of the subsequent samples. In order to minimize this bias, the use of a correction factor [36], the use of labeled reference standard mixtures [38], and automated in-line derivatization were proposed. Moreover, underivatized metabolites can form multiple peaks which might co-elute with other metabolites and hinder the identification of these metabolites. For example, in our chromatograms, partially derivatized fructose eluted closely with citric acid. The concentration of metabolites in biological samples, after extraction, will depend on several factors such as tissue type, extraction method and volume of the extraction

aliquot used for derivatization. Since the metabolome contains metabolites with highly contrasting relative abundances, a delicate balance needs to be maintained between obtaining a sufficient detectable response for the least abundant metabolites and properly completing the derivatization reaction within a moderate time for the highly abundant metabolites. To this end, we have further tuned the derivatization parameters on metabolites extracted from apple tissue.

3.3 Selection of extraction method

The performances of hot pure methanol (HS-PM), two-phase chloroform:methanol:water (HT-CMW), and single-phase chloroform:methanol:water (CS-CMW) were compared for extracting metabolites from apple tissue based on relative extraction efficiency, metabolite recovery, and repeatability. The relative extraction efficiency was expressed as the ratio of the metabolite response to the maximum obtained response for that particular metabolites by all methods. While all three methods were comparable in the number of metabolites extracted, and their relative metabolic profiles (Fig. 5), remarkable differences were observed in the other performance criteria. For most metabolites, HT-CMW had a slightly higher extraction efficiency as compared to HS-PM (Table 4). This was accounted for by the partitioning effect of HT-CMW through an increased enriching of polar metabolites into the water-methanol fraction as compared to chloroform. Even though alanine, threonine, serine, stearic acid and palmitic acid were slightly higher in HS-PM extract, these metabolites had relatively higher RSD in this solvent system. On the other hand, CS-CMW had a very poor extraction efficiency as compared to the other two methods, except for fatty acids.

Temperature increases solubility and diffusivity of the metabolites from the solid matrix into the solvent [2]. The low temperature in CS-CMW might have decreased the solubility of primary metabolites [16] and the subsequent diffusion of metabolites into the extraction solvent. Fatty acids were better extracted by CS-CMW, due to chloroform which is a suitable solvent for apolar metabolites.

RSD value of all metabolites extracted by HS-PM and HT-CMW fell below 15 %, while only 15 % of the metabolites extracted by CS-CMW had a value above this level (Table 4). All methods showed a good repeatability as compared to similar metabolomics studies [20, 21]. Metabolite recovery was calculated from the ratio of the response of metabolites spiked prior and after extraction [18]. This measures the degradation of metabolites due to enzymatic, thermal or chemical reactions during extraction. HS-PM extraction, of all methods, had superior recovery for all metabolic classes suggesting complete inhibition of enzymatic reactions and minimal damage due to heat (Table 5). Similarly, t'Kindt and co-workers found that heated extraction performs better than other methods of enzyme inactivation [16]. In HT-CMW, all metabolites had a recovery higher than 75 % except 2-phosphoglyceric acid, dehydroascorbic acid, stearic acid and glutamine, suggesting the compounds are unstable in this extraction solvent. In comparison, the CS-CMW extraction showed the lowest recovery except for sugars where it performed similar to the other methods. To minimize degradation of highly unstable compounds, cold extraction using MeOH:H_2O:$CHCl_3$ (2.5:1:1) was suggested [9]. Chloroform precipitates proteins and arrests biochemical reactions. Nevertheless, lower recovery of most metabolites in this extraction solvent as compared to hot methanol extraction suggested the incomplete arresting of enzymes.

For recovery test, the tissues were spiked with metabolites which are also present in apple. As a result, the analysis was made by comparing the total responses of the tissue and spiked sample, not only using spiked metabolites. Although we used a single homogenized tissue for

all extraction tests, due to biological variation there might still be some differences between the aliquots. The recovery values higher than one in HS-PM extraction could be a result of this effect. On the other hand, since pre-dissolved metabolites were used for spiking, it could be reasonably assumed that the solubility of metabolites would not cause any variation.

The overall comparison of the three extraction methods is summarized in Table 6. HS-PM is comparable with its performance with HT-CMW, but slightly better in the recovery of metabolites and workability. CS-CMW has the least score in all performance measures. Therefore, HS-PM was selected as the best extraction method for apple tissue.

3.4 Multi-response optimization on a biological sample

To further tune the derivatization parameters taking the concentration of metabolites into account, the second step of optimization was conducted on apple extract. The extraction was conducted using the optimized extraction method described in the previous section. Based on the prediction from the previous experiment, oximation temperature (30 - 50 °C), oximation duration (0.5 - 4 h), silylation temperature (30 - 50 °C), silylation duration (0.5 - 6 h), and 100 µL of MeOX and BSTFA were used as a range of the parameters. Subsequently, BBD with four factors and three levels was applied and multi-response optimization was performed following the same procedure as outlined in section 2.1.2. Because, within the selected range, derivatization parameters had negligible effect on some of the metabolites, only those metabolites that showed a relative standard deviation (RSD) greater than 15 % were considered. Most organic acids and sugar alcohols were in the category of highly stable metabolites.

The derivatization conditions were ranked according to their *D* values, with the most favorable derivatization condition being the one with the highest desirability score (Table 7). Silylation at 37 °C for 1 h had desirability score of 0.5, whereas silylation at 45 °C for 2 h

456 resulted in desirability score of 0.69. While a longer silylation time increased the relative
457 response for GABA, β-alanine, galactinol and glycine, it slightly decreased the relative
458 response of chlorogenic acid (Fig. 6). Higher silylation temperatures (> 45 °C) had negative
459 effect on relative response of asparagine and glutamic acid.

460 For completion of the oximation reaction, 1 h of oximation at low oximation temperature (30
461 °C) was sufficient. However, a similar conversion was attained at shorter duration by
462 increasing the oximation temperature to 50 °C. Long duration at this oximation temperature
463 had a negative effect on dehydroascorbic acid and proline. A short oximation (0.5 h) at high
464 temperature (50 °C) together with a low silylation temperature (30 °C) provided the same
465 desirability score as a long oximation (1 h) at low temperature (30 °C) in combination with a
466 high silylation temperature (45 °C). The total derivatization time was minimized, using total
467 derivatization time as one optimization criteria, while maintaining the best derivatization
468 conditions for most compounds. Increasing the silylation and oximation duration beyond 2 h
469 and 1 h respectively, did not bring any appreciable change on *D*. For example, increasing the
470 silylation duration to 4 h at 45 °C could only change the desirability score by 0.04. This result
471 also suggested, when using higher sample mass, it is possible to increase the oximation
472 temperature and duration without a loss in *D*.

473 Compared under similar range of derivatization parameters, the derivatization patterns of the
474 metabolites both in the standard mixture and apple sample were similar (Fig. 1, 3 and 6). For
475 example, the derivatization parameters had similar effect on dehydroascorbic acid, GABA,
476 asparagine, and glutamic acid. Moreover, the best desirable ranges matched for both samples.
477 This is expected because the standard mixture covered diverse metabolic classes
478 representatives for the polar extracts from the apple tissue.

3.5 Repeatability & stability

In this study, the final method selected for the analysis of apple had shown good repeatability (Table 8). The RSD values were below 10 % for the majority of the compounds, while some less abundant metabolites had RSD values higher than 15 %. Similar repeatability ranges were previously reported for other crops [16, 20, 21]. Derivatized samples need to be stable while they are waiting for GC-MS analysis in the auto sampler. We have assessed the stability derivatized metabolites by injecting same sample over a period of 48 h. The result showed that most compounds were stable over 24 h of derivatization. However, some amino acids such as aspartic acid 3TMS, asparagine, glutamic acid, alanine and valine were highly unstable at 48 h (Fig. 7).

4 Conclusion

As biological tissues are different in their cellular structure, metabolite content and relative abundances it is important to tailor the optimization of GC-MS analysis to fit its purpose. In this study, we optimized the GC-MS protocol for the extraction and analysis of polar metabolites from apple tissue.

The goal in extraction is the complete release of metabolites from the cells while minimizing metabolite conversions. We found pure methanol extraction to be slightly better as compared to the two phase methanol/chloroform/water extraction in terms of introducing less degradation of the extracted metabolites while being comparable in extraction efficiency and repeatability. The methanol extraction was superior to the single phase methanol/chloroform/water extraction in all performance measures.

The metabolome is a complex mixture of widely differing metabolites and finding an optimum set of derivatization parameters through a conventional one-variable-at-a-time based approach is very daunting. By applying DOE and using multi-response optimization based on

a global desirability function it became possible to systematically search for the optimal condition suitable for most metabolites. Using high volumes of derivatization agents (BSTFA and MeOX) was beneficial favoring the complete derivatization of most amino acids. Silylation duration was a critical factor for completion of derivatization. Taken together, oximation at 30 °C for 1 h in combination with silylation at 45 °C for 2 h provided the best derivatization condition for apple. Ensuring sufficient completion of derivatization was helpful for reducing run order based bias and minimize interference from underivatized peaks.

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Table 1. Analyte derivatives identified both in the reference standard mixture and apple tissue with their abbreviations, retention indexes and m/z values used for quantification.

Analyte	Abbreviation	Retention index	m/z used for quantification
Pyruvate TMS	Pyr	1059	174
Alanine 2TMS	Ala	1110	116
Isoleucine TMS	Ile TMS	1205	86
Valine 2TMS	Val	1224	144
Phosphoric acid 3TMS	PO_4	1284	299
Isoleucine 2TMS	Ile 2TMS	1303	158
Proline 2TMS	Pro	1305	142
Glycine 3TMS	Gly	1303	174
Succinic acid 2TMS	Succ	1319	147
Serine 3TMS	Ser	1373	204
Threonine 3TMS	Thr	1400	291
Aspartic acid 2TMS	Asp 2TMS	1432	160
B-alanine 3TMS	β-ala	1437	248
Malic acid 3TMS	Mal	1503	233
Aspartic acid 3TMS	Asp 3TMS	1535	232
5-oxoproline 2TMS	Opro	1536	156
γ-Aminobutric acid 3TMS	GABA	1542	304
Cysteine 3TMS	Cys 3	1571	220
α-Ketoglutaric acid MEOX 2TMS	α-KG	1588	198
Glutamic acid 3TMS	Glu	1633	246
Asparagine 3TMS	Asn	1687	116
Xylitol 5TMS	XylOH	1755	307
Phenyl-β-D-glucopyranoside 8TMS	PβG	1771	361
Glutamine 3TMS	Gln	1789	156
Sorbitol 6TMS	SorOH	1817	319
2-Phosphoglyceric acid 4TMS	2PG	1836	369
Citric acid 4TMS	Cit	1846	273
Quinic acid 5TMS	Quin	1895	345
Fructose MEOX 5TMS peak 1 and 2	Fruc	1910, 1920	307
Glucose MEOX 5TMS peak 1 and 2	Gluc	1937, 1955	319
Dehydroascorbic acid 3MEOX 2TMS	DHAA	1968	316
Palmitic acid TMS	Pal	2050	313
Methyl stearate	Mest	2127	298
Inositol 6TMS	Inos	2131	318
Stearic acid TMS	Stea	2246	341
Cystine 4TMS	Cys	2326	220
3-(4-Hydroxyphenyl)propionic acid 2TMS	HPA	1771	179
Glucose-6-phosphate MEOX 6TMS	G6-P	2374	387
Sucrose 8TMS	Suc	2710	451
Chlorogenic acid 6TMS	Chlo	3291	345

Table 2. Factors and high and low levels used in the three level BBD for derivatization optimization.

Factors (unit)	Low level	High level
Methoxymation temperature (°C)	30	80
Methoxymation duration (h)	0.5	4
Silylation temperature (°C)	30	80
Silylation duration (h)	0.5	6
Methoxyamine hydrochloride (MeOX) volume (µL)	30	125
BSTFA volume (µL)	30	125

Table 3. Coefficients of the second order models for each response variable after backward elimination of non-significant terms and model diagnostics

Compounds	Asp 3TMS	Ile 2TMS	Glu	Gln	Cys	Asp 2TMS	Ile TMS	Asn	Opro	Cys	Succ	Cit	Quin
Intercept	17.31	33.95	2.23	-1.98	0.40	-2.35	-3.40	2.43	5.99	-2.13	22.71	12.26	145.76
A-Oxim-temp		1.17	-0.30	-1.35	0.07		-0.32	-0.46	3.02	-2.27	0.62	0.65	3.54
B-Oxim_dura	1.72	1.49	0.08	-0.65	0.25	-0.03	0.01	-0.11	1.18	-1.82	0.75	0.52	8.14
C-Silyl_temp	-0.30	-0.34	-0.05			-0.01		0.04			-0.25	-0.25	-7.60
D-Silyl_dura	1.80	1.5	0.19		0.10	-1.17	-0.14	0.25		0.45	0.82	0.88	7.83
E-MEOX_amount	4.23	3.14	0.56	0.21	0.04	-0.91	-1.26	0.42	-0.04	0.67	-0.32	-0.49	-23.08
F-BSTFA_amount	4.35	2.29	0.48	0.38	0.34		-1.92	0.69	0.38	0.69	0.10	0.87	25.24
AB		-1.9	-0.38		-0.29			-0.42		-0.91		-0.96	-16.82
AC													-19.85
AD												-0.75	
AE					0.17			-0.31					
AF			-0.28	-0.62	0.15			-0.32	0.54	-0.80	1.45		
BC	-2.07	-1.61						-0.27			-1.00	-1.01	-36.96
BD	-2.36							-0.49					
BE	-2.18		-0.18	-0.22		0.34		-0.40		-0.64			
BF					0.27		-0.64						
CD								-0.29					
CE		2.09	0.27										19.00
CF											1.07	0.71	
DE	3.15	-1.62				-0.32		0.38					
DF											1.20		
EF							-1.03						24.69
A^2		1.25	-0.28	0.51	-0.32			-0.45	-0.54	0.91			
B^2				0.40	-0.30					1.87		0.80	16.32
C^2											-0.86		-23.05
D^2					-0.20		0.44				0.86		21.16
E^2	-4.26	-5.42	-0.60	-0.37	-0.19	0.85	2.93	-0.38	-0.44	-0.86	-1.14	-1.06	-25.90
F^2	-3.51	-2.30	-0.40	-0.35	-0.35	0.45	0.92	-0.77	-0.43	-0.59		-0.95	
Model (P-value)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Lack of Fit (p-value)	0.73	0.3511	0.72	0.80	0.14	0.83	0.73	0.89	0.08	0.36	0.23	0.40	0.62
R-Square	0.79	0.8168	0.80	0.86	0.81	0.85	0.88	0.86	0.91	0.85	0.56	0.58	0.74
Adj R-Square	0.73	0.725	0.74	0.83	0.74	0.81	0.86	0.78	0.89	0.80	0.41	0.43	0.63
Adeq Precision	15.61	15.178	14.38	18.56	13.24	18.04	20.08	15.40	26.18	16.65	9.95	8.29	14.23

Compounds	XylOH	GABA	Pyr	α-KG	2PG	DHAA	Stea	PO_4	G6-P	Fruc	Gluc	Suc
Intercept	7.31	0.83	9.79	6.67	2.18	-2.98	9.59	0.65	2.18		3.15	20.39
A-Oxim-temp	0.39	0.05	0.38	-1.14	-0.40	-1.96	0.76	0.09	0.30	5.00	0.68	-7.22
B-Oxim_dura	0.18	0.11	0.64	0.66	0.04	-1.18	0.76	0.19	0.30	0.97	0.19	0.07
C-Silyl_temp	-0.17	-0.11	-0.12	-0.22	0.08	0.16	1.71	-0.02	0.13	0.25	-0.03	0.41
D-Silyl_dura	0.15	0.18	0.27		0.27		1.08	0.18	0.48	-0.32	0.20	1.47
E-MEOX_amount	-1.08	0.35	-0.24	0.02	-0.18		0.71	-0.01	-0.34	0.65	-0.20	-0.60
F-BSTFA_amount	0.96	-0.18	0.05		0.20		0.78	-0.02	0.19	-0.56	0.31	0.41
AB			-1.55	-1.91	-0.59	-0.04			-0.30	0.77		-5.18
AC							-1.45	-0.15		-0.75		
AD												
AE		-0.12			-0.55				-0.38			
AF										0.54		
BC	-0.83				-0.27	0.79	-0.82	-0.15	-0.24	-1.06	-0.37	-2.06
BD					-0.30							-1.67
BE												
BF						0.72						
CD							0.91					
CE				1.11			1.98		0.20			
CF	0.53		0.35		0.25	-0.57		0.12		0.71	0.33	1.49
DE		-0.09			-0.24							-1.70
DF		0.09	0.45					0.24		1.07	0.29	
EF	1.49	0.27										
A^2		-0.08	-1.17	-1.91		0.54	0.85	0.30		0.98	0.57	-5.50
B^2	1.03	-0.08			0.41							
C^2	-0.81	-0.09	-0.45	-0.76	-0.23							
D^2	0.69		0.53							0.81		
E^2	-1.32	-0.20	-0.61				-1.30	-0.21			-0.28	
F^2								0.22	-0.20			
Model (P-value)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Lack of Fit (p-value)	0.16	0.90	0.36	0.52	0.72	0.02	0.27	0.76	0.16	0.13	0.13	0.41
R-Square	0.74	0.92	0.75	0.61	0.76	0.82	0.80	0.71	0.78	0.79	0.73	0.86
Adj R-Square	0.66	0.89	0.66	0.54	0.67	0.79	0.74	0.61	0.72	0.72	0.66	0.82
Adeq Precision	16.43	21.74	15.35	10.65	13.91	19.62	15.57	12.66	13.91	15.82	12.91	18.12

Table 4. The average extraction efficiency and repeatability of polar metabolites of apple extract (n=5). The relative extraction efficiency was expressed as the ratio of the metabolite response to the maximum obtained response for that particular metabolites by all methods. Experiments: HS-PM (pure MeOH @ 70 °C); CS-CMW (cold single-phase $CHCl_3$:MeOH:H_2O (2:5:2) @ 4 °C); HT-CMW (hot two-phase $CHCl_3$:MeOH:H_2O(1:2:2) @ 70 °C). RSD, relative standard deviation.

Compounds	Extraction efficiency, % (n=5)			RSD (n=5)		
	HS-PM	CS-CMW	HT-CMW	HS-PM	CS -CMW	HT-CMW
Succinic acid	0.94	0.67	0.99	0.85	18.16	3.42
Malic acid	0.83	0.48	0.90	3.47	13.58	5.34
Citric acid	0.85	0.59	0.97	5.22	9.81	6.44
Quinic acid	0.91	0.63	0.97	9.67	9.67	4.15
Phosphoric acid	0.88	0.59	0.98	4.47	10.10	4.24
Alanine	0.85	0.54	0.81	12.43	8.39	5.97
Isoleucine 2TMS	0.90	0.65	0.95	3.69	19.16	7.84
Threonine	0.90	0.50	0.82	8.71	20.07	2.52
Aspartic acid 3TMS	0.89	0.59	0.97	4.69	12.41	2.70
GABA	0.91	0.62	0.98	5.02	8.63	3.29
Glutamic acid	0.90	0.57	0.96	4.28	11.88	2.50
Asparagine	0.93	0.60	0.97	4.57	11.71	2.86
Valine	0.92	0.61	0.98	4.63	9.86	4.92
Proline	0.94	0.60	0.96	4.94	10.67	3.14
Glycine	0.88	0.64	0.90	8.36	6.16	8.27
Serine	0.83	0.46	0.70	13.73	17.34	4.31
Beta alanine	0.85	0.58	0.97	6.76	8.34	5.71
Sorbitol	0.91	0.56	0.97	5.44	12.06	3.32
Xylitol	0.91	0.60	0.97	4.83	8.75	5.46
Allo-Inositol	0.89	0.59	0.97	3.79	9.52	3.52
Stearic acid	0.66	0.88	0.57	7.93	10.39	2.85
Palmitic acid	0.70	0.88	0.60	7.98	8.77	6.91
Glucose-6-phosphate	0.86	0.56	0.98	7.29	9.21	3.11
Sucrose	0.88	0.48	0.94	4.34	12.79	4.71
Fructose	0.90	0.51	0.96	5.72	11.82	4.14
Glucose	0.90	0.54	0.96	5.55	11.42	4.06
Chlorogenic acid	0.82	0.44	0.95	7.69	14.56	7.34

Table 5. Average recovery of metabolites during three different extraction methods for apple tissue (n=6). Metabolite recovery was calculated from the ratio of the response of metabolites spiked before and after extraction. Experiments: HS-PM (pure MeOH @ 70 °C); CS-CMW (cold single-phase $CHCl_3$:MeOH:H_2O (2:5:2) @ 4 °C); HT-CMW (hot two-phase $CHCl_3$:MeOH:H_2O(1:2:2) @ 70 °C).

	Recovery, %		
Compounds	HS-PM	CS-CMW	HT-CMW
Pyruvic acid	1.07	0.59	0.85
Succinic acid	1.06	0.61	0.81
Malic acid	1.08	1.03	1.07
Citric acid	0.98	0.68	0.81
Quinic acid	1.04	0.67	0.83
Dehydroascorbic acid	1.09	0.60	0.68
α-ketoglutaric acid	0.96	0.58	0.75
Phosphoric acid	1.08	0.95	1.08
Alanine	1.10	0.61	0.81
Isoleucine 2TMS	1.06	0.61	0.80
Threonine	1.02	0.59	0.78
Aspartic acid 3TMS	1.13	0.78	0.94
GABA	1.06	0.62	0.80
Glutamic acid	1.08	0.77	0.93
Asparagine	1.06	0.91	1.03
Glutamine	1.05	0.94	0.63
Sorbitol	1.07	1.03	1.08
Xylitol	1.06	0.62	0.80
Cysteine	1.09	0.59	0.86
Stearic acid	1.04	0.64	0.15
2-phosphoglyceric acid	0.89	0.69	0.54
Glucose-6-phosphate	0.95	0.62	0.78
Sucrose	1.06	1.04	1.04
Fructose	1.07	1.05	1.08
Glucose	1.08	1.05	1.08

Table 6. Summary of the comparison of three extraction methods tested for apple tissue. The extraction efficiency and recovery of each metabolites were combined into single value by using geometric mean. Experiments: HS-PM (pure MeOH @ 70 °C); CS-CMW (cold single-phase $CHCl_3$:MeOH:H_2O (2:5:2) @ 4 °C); HT-CMW (hot two-phase $CHCl_3$:MeOH:H_2O(1:2:2) @ 70 °C). RSD, relative standard deviation.

Extraction method	Extraction efficiency	Recovery	Repeatability RSD (n=5)	Workability
HS-PM	0.87	1.05	6.2	+++
CS-CMW	0.58	0.74	11.7	+++
HT-CMW	0.90	0.80	4.2	++

Table 7. Desirability score of different derivatization conditions for apple extract.

Oximation temperature (°C)	Oximation duration (h)	Silylation temperature (°C)	Silylation duration (h)	Desirability
30	1.0	45	2.0	0.69
50	0.5	30	3.0	0.68
30	1.0	45	1.0	0.57
30	1.5	37	1.0	0.50

Table 8. Repeatability of metabolites extracted and derivatized from homogenized apple tissue in eight different tubes. The repeatability is expressed as the relative standard deviation (RSD).

Compounds	Repeatability, RSD (n=8)
Succinic acid	18.1
Malic acid	2.4
Citric acid	4.8
Quinic acid	1.9
Phosphoric acid	2.2
Alanine	21.7
Aspartic acid 3TMS	4.7
GABA	3.1
Glutamic acid	6.6
Asparagine	7.8
Valine	10.0
Glycine	9.1
Beta alanine	7.8
Sorbitol	2.6
Xylitol	2.0
allo-Inositol	1.5
Palmitic acid	9.3
Glucose-6-phosphate	2.7
Sucrose	3.0
Fructose	2.8
Glucose	3.1
Chlorogenic acid	11.2
Epicatechin	4.5

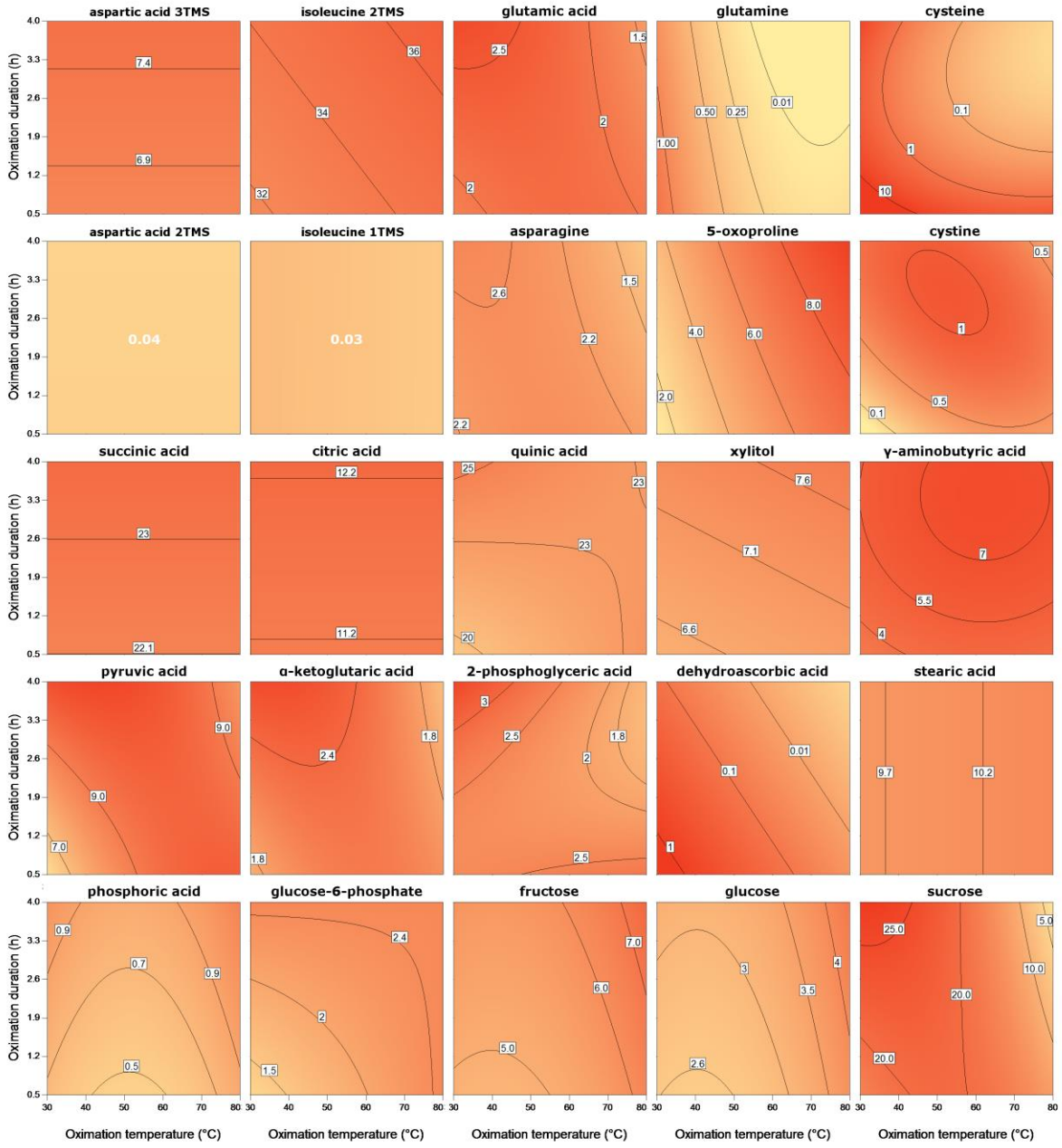

Fig. 1. Contour plots showing the effect of oximation temperature (30-80 °C) and oximation duration (0.5-4 h) on relative response of metabolites and derivatization by-products.

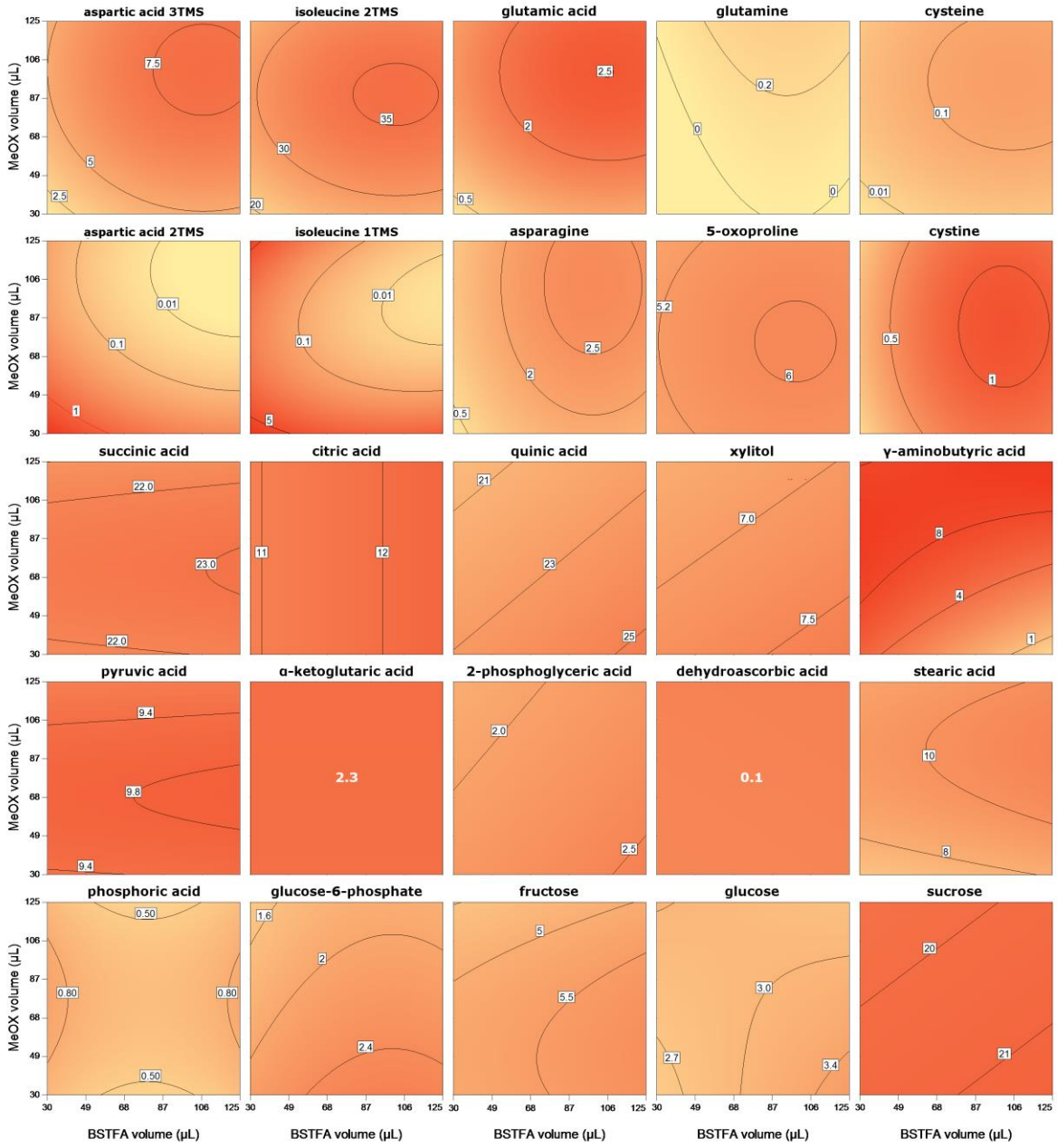

Fig. 2. Contour plots showing the effect of BSTFA (30-125 µL) and methoxyamine hydrochloride dissolved in pyridine (30-125 µL) on relative response of metabolites and derivatization by-products.

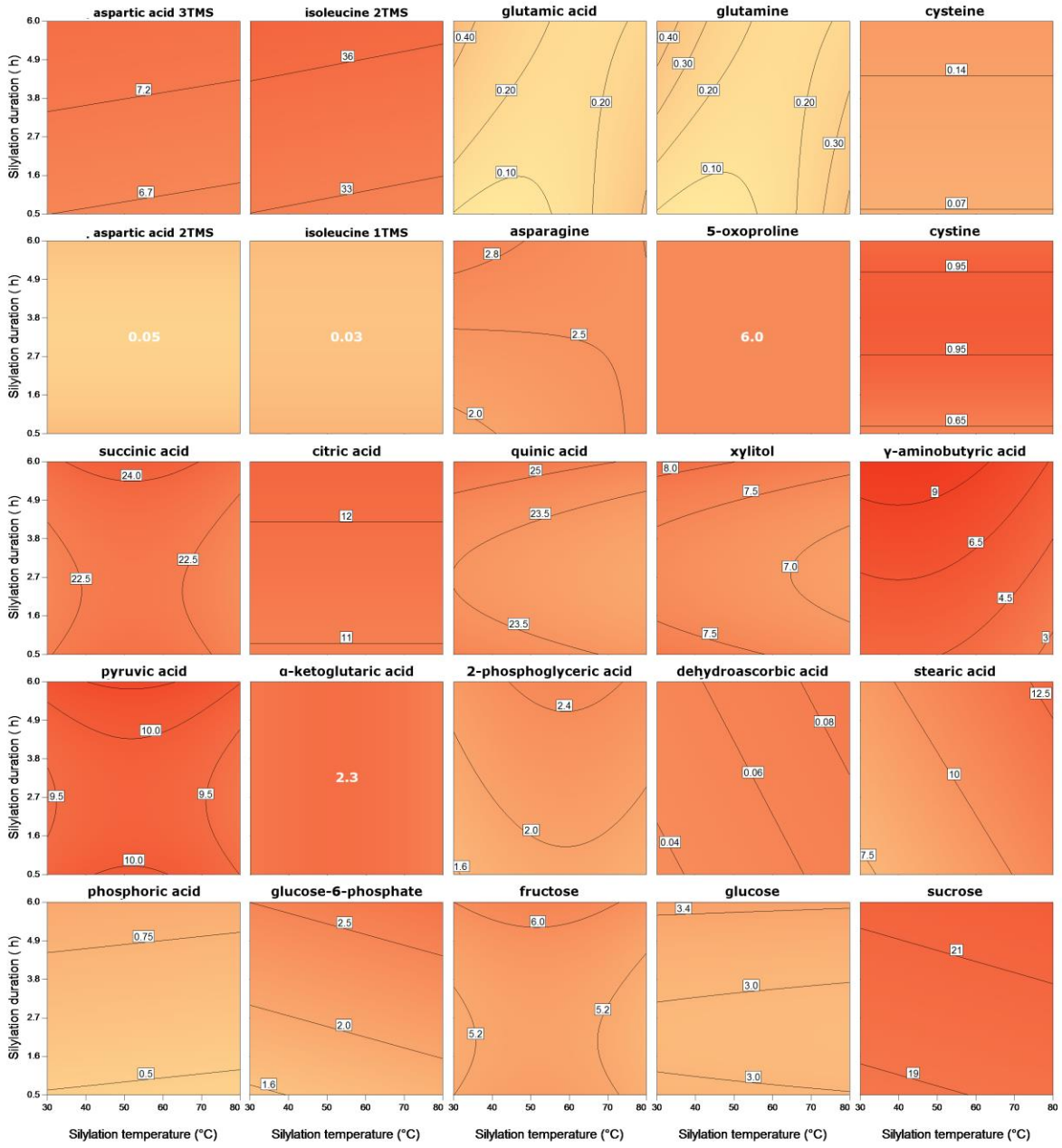

Fig. 3. Contour plots showing the effect of silylation temperature (30-80 °C) and silylation duration (0.5-6 h) on relative response of metabolites and derivatization by-products.

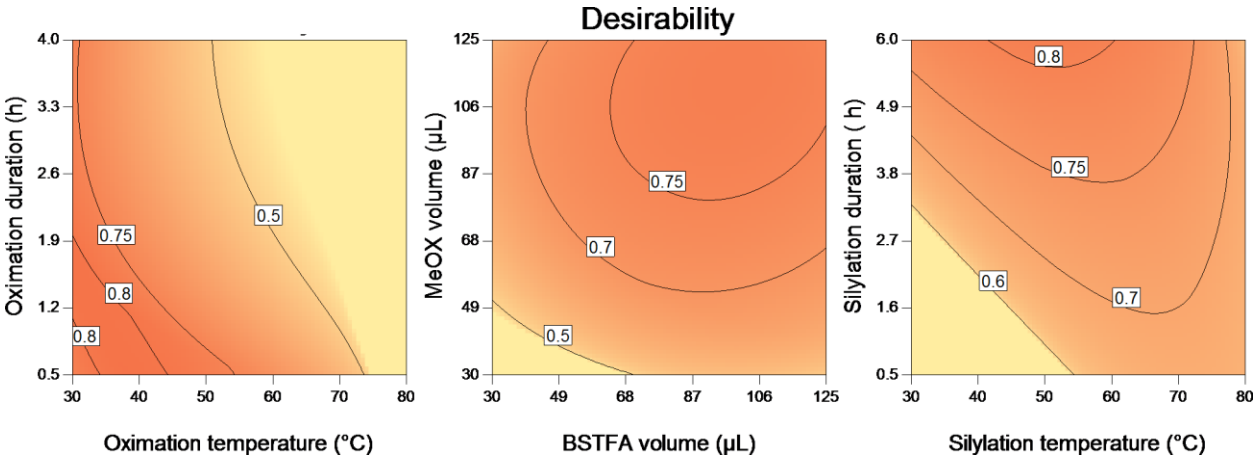

Fig. 4. Contour plots showing the global desirability representing the combined individual desirabilities of all metabolites within the range of oximation temperature (30-80 °C) and oximation duration (0.5-4 h); BSTFA volume (30-125 µL) and MeOX volume (30-125 µL); and silylation temperature (30-80 °C) and silylation duration (0.5-6 h).

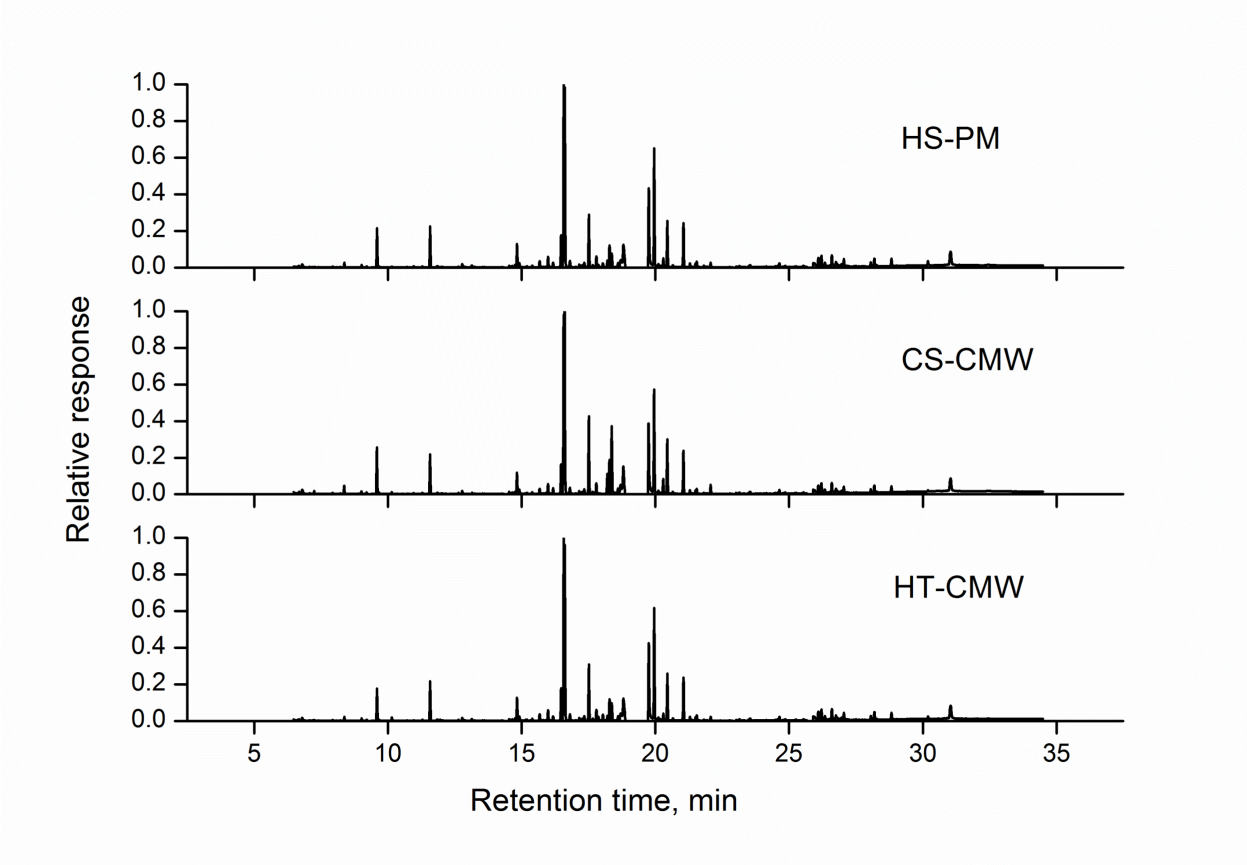

Fig. 5. Chromatograms of apple tissue extracted by three different solvents. The relative response is expressed as relative to the maximum peak intensity for a particular method. HS-PM: 100 % MeOH @ 70 °C; CS-CMW: cold-single phase $CHCl_3$:MeOH:H_2O (2:5:2) @ 4 °C; HT-CMW: hot two-phase $CHCl_3$:MeOH:H_2O(1:2:2) @ 70 °C.

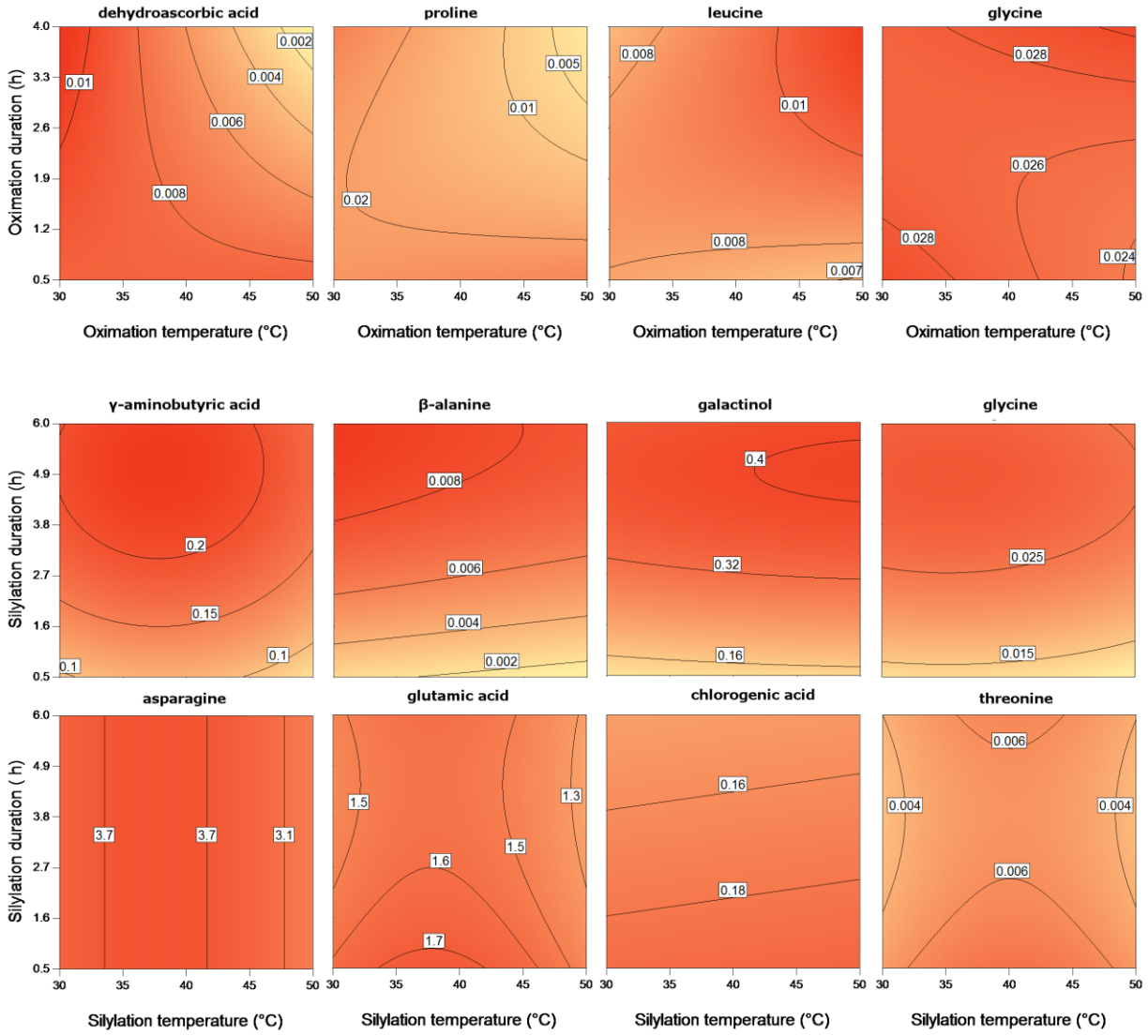

Fig. 6. Contour plots showing the effect of oximation temperature (30-50 °C) and oximation duration (0.5-4 h), and silylation temperature (30-50 °C) and silylation duration (0.5-6 h) on relative response of metabolites extracted from apple tissue.

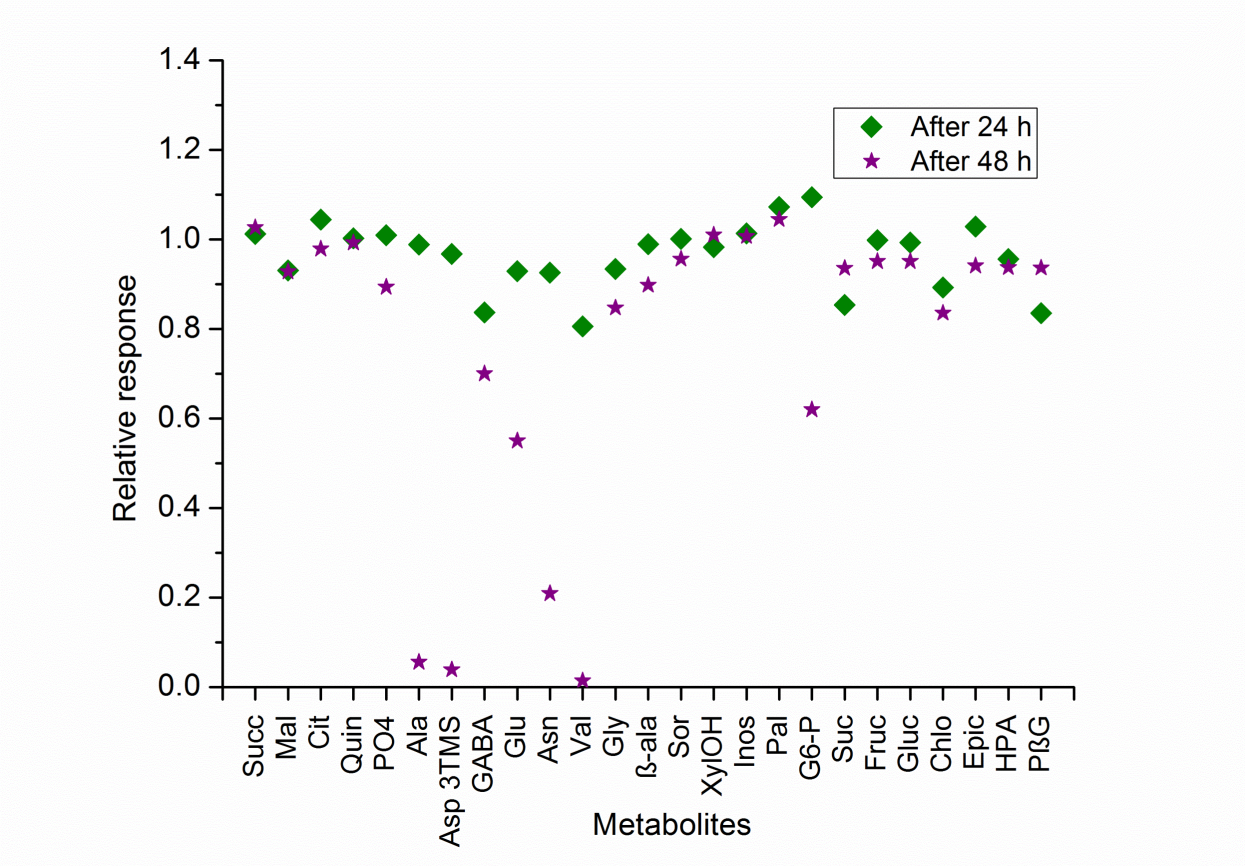

Fig. 7. The stability of derivatized metabolites kept at room temperature. The homogenized derivatized samples were injected (n=3) immediately after derivatization, after 24 h , and after 48 h. The mean values of samples injected after 24 h and 48 h were normalized by the mean response immediately after derivatization.